Biomonitoring Brevetoxin Exposure in Mammals Using Blood Collection Cards

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A method has been tested in laboratory mice to monitor for the presence of brevetoxins in blood after exposure. The use of blood collection cards is an adaptation of a method employed for routine diagnostic and genetic testing of newborns. Blood is collected and applied to a 0.5-inch diameter circle on a specially prepared blood collection card and allowed to dry. The blood spots are then extracted and the presence of toxin activity is first screened using a high throughput receptor binding assay. Positive samples are then examined for specific brevetoxin congeners by liquid chromatography–tandem mass spectrometry. Preliminary experiments tested the efficiency and linearity of toxin extraction from blood spiked with brevetoxin-3 (PbTx-3). Blood from treated mice was tested for the presence of brevetoxin at different times following exposure to a sublethal dose (180 µg/kg PbTx-3). Brevetoxin activity determined by receptor assay increased to 25 ± 7.4 nM PbTx-3 equivalents within 4 hr after exposure and was still detectable in three of four animals 24 hr after exposure. Tandem mass spectrometry provided confirmation of PbTx-3, which also increased for the time points between 0.5 and 4.0 hr exposure. However, PbTx-3 was not detected at 24 hr, which suggested the formation of a biologically active metabolite. We anticipate that this approach will provide a method to biomonitor brevetoxins in living marine resources (e.g., finfish), protected species, and humans. *Key words*: blood, brevetoxin, harmful algae, red tide. *Environ Health Perspect* 109:717–720 (2001). [Online 5 July 2001]
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The need for definitive toxin identification is critical in the cases of unusual mortality events that are associated with harmful algal blooms. Florida red tides have been known to be associated with marine animal mortality events since 1844 (*1*). Fish are the primary organisms affected and, depending upon the severity of the red tide, over 90 different finfish species have been identified in red tide-associated mortality (*1–7*). More severe events have included mortalities of turtles (*3*), seven species of birds (*1*), bottlenose dolphins (*3*), and manatees (*8*). Humans are susceptible to adverse effects through direct inhalation of aerosolized brevetoxins during bloom events or through consumption of shellfish that have accumulated brevetoxins. A report on the toxicity of shellfish during red tide events was first documented in 1884 (*1*), and respiratory irritation was recorded as early as 1917 (*2,9*).

Functional assays have been used to monitor the presence of brevetoxin activity predominantly in shellfish. Mouse bioassays have been used to monitor brevetoxin activity in shellfish as part of a management program for the harvest of shellfish in Florida waters (*10,11*). Receptor-based assay and radioimmunoassay also have been used to monitor activity in shellfish and affected consumers (*12*). However, direct measurement of the toxin by chemical analysis has only recently been applied to the toxin. In November 1999, tissue extracts from bottlenose dolphins, which were associated with a prolonged red tide on the gulf coast of Florida, were determined by receptor assay to have high levels of brevetoxin activity and confirmed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) to contain brevetoxin-3 (PbTx-3) (*13*).

However, the relationship between environmental brevetoxin exposure and its adverse effects in wildlife and humans is poorly defined. Our present knowledge of the amount of toxin that causes adverse effects in animals or in humans (effect level) is incomplete. Accordingly, health officials cannot determine the gravity of harmful algal bloom incidents without more accurate human or animal exposure information. Biomonitoring provides direct measurement of toxins in human or animal tissue collected from living subjects as a means to assess exposure. Common fluids, cells, and tissues used for biomonitoring include blood, urine, hair, and circulating blood cells, or biopsy tissue, with blood being the most commonly used tissue in humans.

In this article we describe the application of a method based on the blood card collection method used by the U.S. Centers for Disease Control and Prevention's Newborn Screening Program (Atlanta, GA). In this study, we applied the blood collection card sampling method to the measurement of brevetoxin using a two-tiered analysis that couples a high throughput microplate receptor assay (*14*) and LC-MS/MS.

Methods

The whole blood used in the spiking experiments included rat blood (Harlan Bioproducts, Indianapolis, IN), dolphin (*Tursiops truncatus*), or menhaden (*Brevoortia tyrannus*) blood. Dolphin blood was provided by W. McFee of the Marine Mammal Program at the NOAA Center for Coastal Environmental Health and Biomolecular Research (Charleston, SC). Menhaden blood was collected with heparinized capillary tubes from the tail vein of animals anesthetized with 150 µg/L MS-222 (Sigma Chemical Company, St. Louis, MO). The blood was spiked with 600, 60, and 6 nM PbTx-3 (Calbiochem, San Diego, CA), and control spots contained an equal volume of the methanol vehicle. We applied 100 µL of spiked blood to each circle on the blood collection cards (Figure 1). Cards were allowed to dry in a cool, dark place overnight. After the spots were dry, the cards were stored at –20°C in airtight plastic bags (VWR Scientific Products, Suwanee, GA) containing desiccant packages (Multisorb Technologies Inc., Buffalo, NY) and humidity cards (Multisorb Technologies Inc.) until use.

Entire blood spots were separated from blood collection cards and extracted overnight in 2 mL methanol for use either in the receptor binding assay for brevetoxin or

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for analysis by LC-MS/MS. The extracts from the spots were blown to dryness using N_2 gas and resuspended in 100 µL of assay buffer for the receptor binding assay or 100 µL methanol for LC-MS/MS.

Receptor binding assay. We performed receptor binding assays in 96-well plates in a buffer consisting of the following: 50 mM HEPES (pH 7.4), 130 mM choline chloride, 5.5 mM glucose, 0.8 mM magnesium sulfate, 5.4 mM potassium chloride, 1 mg/mL BSA, and 0.02% Emulphor-EL 620 (*14*) (all reagents from Sigma Chemical Company, except for Emulphor, which was from GAF, New York, NY). The final per-well assay volume was 210 µL, composed of 35 µL [3H] PbTx-3, 35 µL standard or sample, and 140 µL rat brain membrane preparation (1 mg protein/ml). PbTx-3 standards ranged from 10 pM to 10 µM. Dried blood spot extracts in glass ampoules were resuspended in 100 µL (the original spot volume) using assay buffer and sonicated briefly. We incubated them for 1 hr at 4˚C. We then filtered brain membrane homogenates onto a 96-place glass fiber filter mat (Perkin Elmer Life Sciences, Gaithersburg, MD). Each sample was washed four times with ice cold assay buffer. The filter mat was dried on a slide warmer (60˚C) for 15 min and then saturated with solid scintillant (Perkin Elmer Life Sciences) by heating until the filter mat became transparent. The mat was cooled and then counted on a 1450 Microbeta (Perkin Elmer Life Sciences) scintillation counter.

LC-MS/MS. We conducted chromatographic separations using an Agilint (Palo Alto, CA) HP-1100 HPLC system. The solvent delivery apparatus employed a binary pump coupled to a high-pressure mixing system. Solvents were vacuum degassed in line. The separations involved a water:methanol elvant scheme with 0.1% (v/v) trifluoroacetic acid added to both phases. Separations were done on a Vydac 201TP (C-18) 2 mm × 100 mm column (The Separations Group, Hesperia, CA). The column was protected with a Vydac 201TP 2 mm guard column. We injected samples into the mobile phase flow with the HP-1100 autoinjector. Separations involved a programmed gradient using a flow rate of 200 µL/min with the following steps: *a*) 5 min 50% methanol; *b*) 50–95% methanol over 20 min; *c*) 5 min 95% methanol; *d*) 95–50% methanol over 5 min; *e*) 5 min 50% methanol.

All mass spectrometric (MS) analyses were conducted on a SCIEX API-III+ (Thornhill, Ontario, Canada) triple quadrupole mass spectrometer using atmospheric pressure chemical ionization (APCI). The curtain gas, nebulization, and auxiliary gas flows for the MS ion source were obtained from the boil-off from the house liquid nitrogen supply. For all experiments, we adjusted source ion optics to accomplish desolvation of ions while minimizing fragmentation of analyte ions in the inlet region of the mass spectrometer. We controlled the first quadrupole so that only PbTx-3 pseudomolecular ions were passed to the collision cell in the second quadrupole region used for fragmentation. The resulting PbTx-3 fragments were directed through the third quadrupole to the MS detector. Quantitation was based on integrated chromatographic peak areas for the distinctive PbTx-3 fragment ions at 724 and 878 *m*/*z*. Analysis of the origin of the 724 and 878 masses at 26-min retention confirmed that they were derived from an 896 parent.

Mouse exposure. Female ICR (CD-1) mice, 18 to 20 g, were obtained from Harlan Sprague Dawley (Indianapolis, IN). Food and water were given *ad libitum*. Mice were kept 24 hr before dosing. We injected 20 mice intraperitoneally (IP) with either 180 µg/kg PbTx-3 (LD_{50} = 94 µg/kg) and 4 mice with 1.66% methanol in PBS. Each mouse was used one time; blood was collected from 4 mice per time point (30 min, 1 hr, 2 hr, 4 hr, and 24 hr). At the appropriate time point 4 mice were anesthetized with 2.0 mg ketamine (Parke-Davis, Morris Plains, NJ) and 0.02 mg Prom ACE (Aveco, Fort Dodge, IA) in a volume of 100 mL of phosphate-buffered saline. After anesthetization, we collected blood one time from each mouse by a cardiac puncture to the left ventricle with a lithium heparinized 1 mL syringe. We applied 100 µL blood to each spot on the blood collection card. The handling of blood spot cards, extraction, and testing proceeded as stated previously.

Results

We performed initial experiments to assess the efficiency of extraction of brevetoxin from the blood spots. After extraction with methanol, the paperbound blood spot remained red and the methanol extract was clear with a light green tint (Figure 2). Dolphin blood was spiked with [3H]-PbTx-3 before being applied to the card; 84 ± 2.4% of the radioactivity applied was in the methanol extract. Residue on the paper was treated with peroxide to eliminate color, and 16% of the tritium remained on the card.

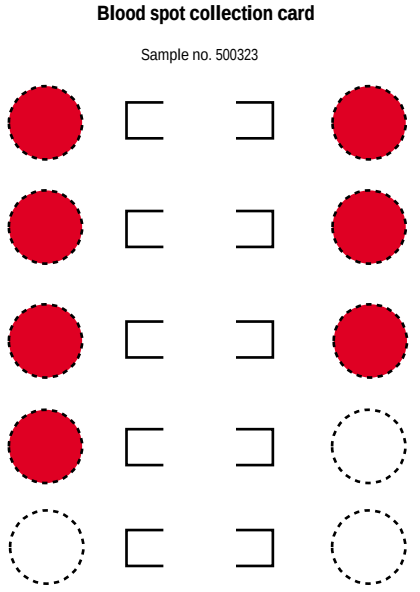

Figure 1. Sample of filter paper card used for collecting blood spots. Blood (100 µL) is spotted on specially prepared cotton fiber filter paper cards. The blood is allowed to dry overnight and stored at –20˚C until use.

Figure 2. Blood spot extraction. Whole blood spots are extracted overnight in 2 mL methanol. The methanol containing extracted PbTx-3 is removed and blown to dryness with N_2 for analysis on receptor-binding assay or by LC-MS/MS. This extraction method provides a quick and simple method to separate brevetoxin from the blood matrix.

Figure 3. Spike recovery of brevetoxin from blood collection cards. The extraction of brevetoxin from blood collection cards displayed brevetoxin activity that was proportional to the amount of PbTx-3 spiked in the blood. Whole rat blood was spiked with 6 nM, 60 nM, and 600 nM PbTx-3. Three spots were extracted and run on the receptor-binding assay for brevetoxin from a single experiment. Both x- and y-axes are shown in the log scale.

We next determined whether we could measure brevetoxin activity in blood extracted from the card using receptor assay.

We added PbTx-3 to whole rat blood to achieve concentrations of 6, 60, and 600 nM. We extracted blood spots and measured toxin activity at a 1/6 dilution using the microplate receptor assay. We detected brevetoxin activity at each of the three doses, as well as the amount of activity, which we determined via receptor assay by fitting the unknown to a standard curve. The amount of PbTx-3 detected was proportional to the amount of toxin applied to the blood (Figure 3); for a 60 nM spike, 51 ± 5 nM was measured (n = 4). The next step was to determine whether brevetoxin could be detected in the blood of mice treated with a sublethal dose of brevetoxin.

Brevetoxin activity was detectable by receptor assay in two of the four mice that were scarified for the 30 min postexposure time point. The receptor assay detected brevetoxin in three of four mice for both the 1 hr and 2 hr postexposure groups. By 4 hr after exposure, brevetoxin activity was observed in all four of the treated animals at a level of 25 ± 7.4 nM PbTx-3 equivalents (Figure 4). At 24 hr after exposure, brevetoxin was detectable in three of four animals. We next examined whether we could confirm the presence of PbTx-3 by LC-MS/MS.

The first step was to determine whether we could identify PbTx-3 in blood by LC-MS/MS. We compared LC-MS/MS analysis of blood extract derived from a PbTx-3–treated mouse with a PbTx-3 standard observed under identical MS and MS/MS conditions. Both the parent ion of PbTx-3—896 Da—and two daughter ions—878 Da and 724 Da—all with identical retention times, were monitored to confirm the presence of PbTx-3. Specific monitoring of the 724 Da fragment provided the least interference from the bulk of the matrix material observed in Figure 5, within the high methanol portion of the HPLC elvant scheme. We observed similar chromatographic patterns for both the standard and the mouse blood extract. We next examined the blood of animals identified as positive by the receptor assay screen for each dose using LC-MS/MS.

We analyzed blood extracted from positive tested animals for PbTx-3 parent ion and the two daughter fragments. Figure 6 shows the diagnostic 724 Da fragments for no treatment and for 0.5, 1.0, 2.0, 4.0, and 24 hr exposure. Both the parent ion and the two fragments were present; however, the PbTx-3 724 Da fragment provided the cleanest peak, which increased between 0.5 hr and 4 hr. The parent PbTx-3 ion and the two daughter fragments could not be detected at the 24-hr time point.

Discussion

The goal of this study was to develop a rapid and efficient sampling method that could be applied to monitor brevetoxin exposure in marine animals and potentially humans. We have adapted the method used by the Centers for Disease Control and Prevention to collect and store blood for analysis of substances and detection of diseases. During the initial steps in adapting this method for toxin detection, we were concerned with the feasibility of extracting brevetoxin from the collection cards. Accordingly, we conducted preliminary experiments to evaluate recovery of toxin from the cards. We determined that brevetoxin could be extracted from the dried spots from the collection cards and that there was a linear recovery of the toxins. Not only did the extraction method involving the dried blood spots prove to be an easy way to separate brevetoxin from the blood matrix,

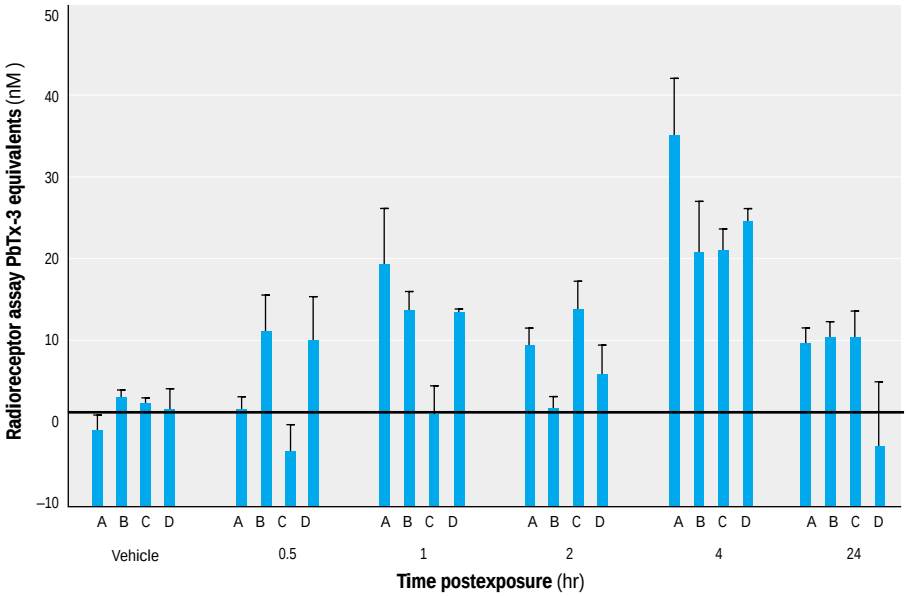

Figure 4. Receptor assay results from mouse exposure. Mice were exposed to 180 µg/kg PbTx-3, and blood was collected from mice before exposure or at 0.5, 1.0, 2.0, 4.0, or 24 hr after exposure. Four mice (A,B,C,D) were used for each time point. Receptor-binding activity showed an increase in brevetoxin activity up to 4 hr. Activity decreased but remained detectable at 24 hr. Results shown are for four animals at each time point from a single experiment. Values given are mean + SE from triplicate blood spots from one animal.

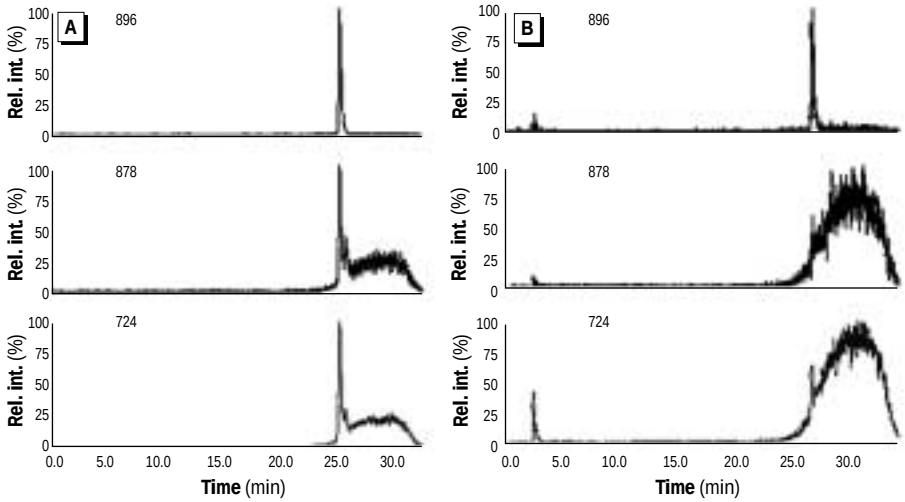

Figure 5. LC-MS/MS conformation of PbTx-3 in mouse blood. (*A*) shows the LC-MS/MS chromatogram of 0.1 µg of a PbTx-3 standard at 724, 878, and 896 *m/z*. (*B*) shows the LC-MS/MS chromatogram for an extracted blood spot taken from a mouse treated with PbTx-3 at 724, 878, and 896 *m/z*. The PbTx-3 parent and its MS/MS fragments are detected at 26 min retention time. Additional mass is also evident at longer retention times represent the bulk material released at the high methanol portion of the chromatograhic run as well as coalescing of the highly ionizable solvents. Of note is that different compounds ionize at different intensities so that relative intensities (Rel. int.) are not really comparable.

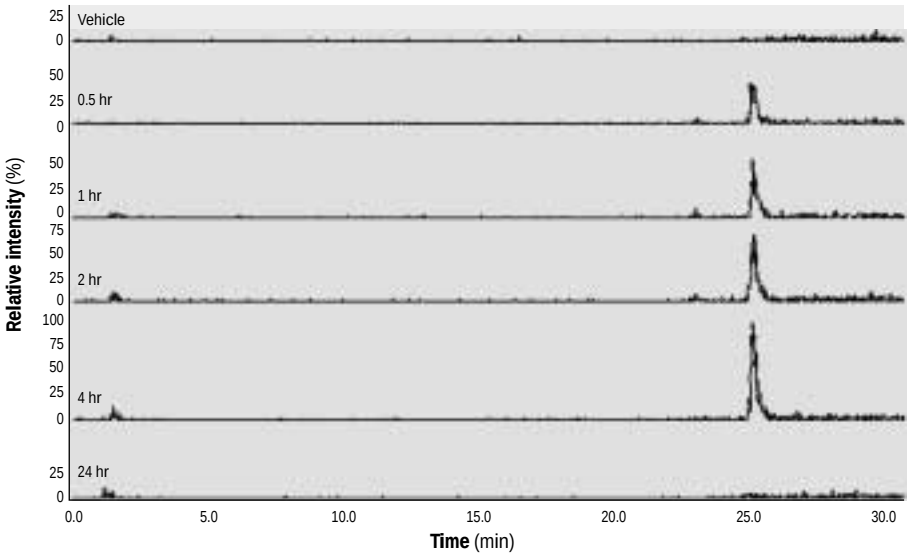

Figure 6. LC-MS/MS results from mouse exposure. LC-MS/MS analysis of the time course exposure in mice to PbTx-3 showed an increase of PbTx-3 in the blood up to 4 hr as displayed by the 724 Da ion. LC-MS/MS was unable to detect PbTx-3 at the 24-hr time point.

thereby minimizing clean-up, but it also allowed the extracted toxin to be concentrated easily before application to the assay.

The distribution of brevetoxin in the blood or serum of laboratory rodents has been examined following several different exposure paradigms. Poli et al. (*15*) determined that 90% of [3H] PbTx-3 was eliminated from the rat serum within 1 min of administration into the cranial vena cava. However, when rats were given an oral dose of [3H] PbTx-3, the toxin remained in the serum up to 192 hr after administration, with maximum concentration found at 48 hr (*16*). Our time exposure studies have indicated that brevetoxin activity can be found in the blood of mice up to 24 hr after intraperitoneal exposure. Both LC-MS/MS analysis and receptor-binding data reflect an increase in PbTx-3 up to 4 hr after exposure. However, the receptor-binding assay continued to detect brevetoxin activity at 24 hr, whereas LC-MS/MS analysis no longer found the PbTx-3 congener at this time period.

The method of administration of the toxin could allow for different clearance rates of the toxin among these studies. An injection of toxin directly into the blood stream as performed by Poli et al. (*15*) can allow for quick distribution into the tissues and out of the blood. Oral administration as performed by Cattet and Geraci (*16*), intratracheal instillation as preformed by Benson et al. (*17*), or intraparenteal administration as performed in this study may keep the toxin sequestered in other compartments and allow the toxin to be released slowly into the blood and hence detectable for a longer time.

The differences in the detection of brevetoxin by the two methods at the 24-hr time point may be attributed to metabolism of PbTx-3. The receptor-binding assay was able to detect composite activity of the different congeners or metabolites of brevetoxin, which actively compete for the receptor, whereas LC-MS/MS analysis was limited to the detection of the PbTx-3 congener. The understanding of which metabolites remain in circulation and their biologic effects is important for the identification of longer-lasting biomarkers of exposure as well as a more complete understanding of the adverse effects of environmental exposures of brevetoxins on marine animals and humans.

Current studies are directed at determining the applicability of this method to animals exposed in a natural setting. It is critically important to measure the levels of brevetoxin in the blood and how they relate to toxicity in order to determine effect levels for brevetoxin of animals that are frequently exposed. The blood collection card method provides a simple, reliable way to collect and store samples directly in either the field or the clinic. It also provides a solid phase for sample clean-up. Receptor-based assay of blood extracts provides a suitable way to screen positive samples, and LC-MS/MS provide a suitable way to identify definitively the specific brevetoxin congeners several hours after exposure. Identification of the toxin at later exposure times will likely require identification of brevetoxin metabolites.

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